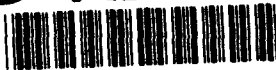


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**MONOCLONAL ANTIBODY DETECTION OF CHLORINATED BENZENES ON  
CONTAMINATED SEDIMENTS**

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27 May 1993

Annual Technical Report for the Period 5/1/92 - 4/30/93

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## TABLE OF CONTENTS

RESEARCH OBJECTIVES .....	1
STATUS OF THE RESEARCH EFFORT .....	2
Significant Accomplishments .....	2
Progress .....	2
Antibody Production .....	2
Antibody Selectivity .....	4
Adaptation of ELISA to Sediments .....	4
Soil Samples and Standards .....	6
PUBLICATIONS RESULTING FROM THE RESEARCH EFFORT .....	7
PROFESSIONAL PERSONNEL .....	8
INTERACTIONS .....	8
Papers presented .....	8
NEW DISCOVERIES .....	9
Patent Applications .....	9
Additional Research Avenues .....	10
RELATED PROPOSALS .....	11
AASERT .....	11
DEPSCoR .....	11
PROJECT SCHEDULE .....	12
EXPERIMENTAL RESULTS - 5/20/93 .....	13
EXPERIMENTAL RESULTS - 5/20/93 .....	14
EXPERIMENTAL RESULTS - 1/29/93 .....	15
EXPERIMENTAL RESULTS - 5/27/93 .....	16

**Monoclonal Antibody Detection of Chlorinated Benzenes on Contaminated Sediments  
AFOSR 91-0236**

**RESEARCH OBJECTIVES**

1. Generate known chlorinated benzene (CB) concentrations on activated carbon. These references will be used as a standard with which to calibrate the tests.
2. Produce murine monoclonal antibodies to three CB: chlorobenzene, 1,2-dichlorobenzene, and 1,3-dichlorobenzene, using the corresponding aniline compounds (4-chloroaniline, 3,4-dichloroaniline, and 2,4-dichloroaniline, respectively).
3. Adapt the ELISA for sediments. The *Enzyme-Linked ImmunoSorbent Assay* utilizes antibodies to quantify the presence of certain chemicals (CB, in this case) using an enzyme-mediated color marker. ELISAs are well established for aqueous samples: a significant effort in this research will be targeted to modifying ELISA techniques to a solid phase sample.
4. Establishing the quantitative relationship between color development of the substrate and concentration of CB sorbed to the activated carbon (i.e. developing a standard curve for mg CB/g carbon).
5. Compare accuracy and precision of immunoassay test to extraction/chromatography techniques.

The objectives of the second year were to produce antibodies to chlorobenzene, 1,2-dichlorobenzene, and 1,3-dichlorobenzene, to make sediment reference samples, and to begin developing the protocol for applying the immunoassay directly to sediments using the anti-CB antibodies.

## STATUS OF THE RESEARCH EFFORT

A Gantt chart is provided on page 10 to graphically present the schedule of the research project. Solid lines and markers indicated completed tasks or portions of tasks. Open markers and dotted lines indicate incomplete tasks.

### Significant Accomplishments

- A modification to the standard ELISA procedure which eliminates the need for extraction of sorbed pollutants was demonstrated. The procedure currently analyzes for dinitrobenzene sulfonate on granular materials.
- Monoclonal antibodies were produced which are specific to 4-chloroaniline (analog for chlorobenzene), 3,4-dichloroaniline (analog for 1,2-dichlorobenzene), and 2,4-dichloroaniline (analog for 1,3-dichlorobenzene).

### Progress

#### **Antibody Production**

Murine hybridoma cell lines were received from the Cell and Immunology Core (CIC) at the University of Missouri-Columbia. These cell lines resulted from the fusion of the sacrificed mouse splenocytes and the NS01 myeloma fusion partner. The mouse was sacrificed when the antibody titer of test bleeds exceeded 50,000. The fusions were conducted in a polyethylene glycol solution. Three types of fused cells were generated: splenocyte-splenocyte, myeloma-myeloma, and splenocyte-myeloma. Only a small percentage of cells were the desired splenocyte-myeloma fusion.

Fused cells were grown in 96 well plates: viable hybridomae (i.e. splenocyte-myeloma) were selected using hypoxanthine aminopterin thymidine (HAT) media. Supernatant was collected and tested by ELISA. Hybridomae which secrete the desired antibodies were transferred to Mossman's laboratory.

Monoclonal antibodies are the preferred for analytical use because they are of a single type (e.g. IgG) and give highly reproducible data. The hybridoma cell lines were subcloned by limiting dilutions. Approximately 40-50 clones were identified for each fusion. The antibodies secreted by clones into the supernatant were tested by ELISA for selection. Desired clonal cell lines (see Table 1) were increased in volume from 100  $\mu$ l to 20 ml. At least 500 ml of supernatant were collected for each clone line. Both serum and serum-free media were used.

**Table 1**

<u>Antibodies to</u>	<u>Number of clonal cell lines</u>
4-chloroaniline	6
2,4-dichloroaniline	5
3,4-dichloroaniline	11

Backup cultures of the clones were frozen in liquid nitrogen at CIC.

The collected antibodies were concentrated for ease of use and stability in long term storage. The supernatants from the 4-chloroaniline clones were concentrated by ammonium sulfate precipitation, and dialysis. It appears that there was some irreversible denaturation and loss of antibodies, so subsequent concentrations will use ultrafiltration concentration by

molecular weight cutoff. After concentration, the antibody solutions were frozen ( $-5^{\circ}\text{C}$ ) for storage in the laboratory.

### **Antibody Selectivity**

The antibodies to 4-chloroaniline (4-CA) were tested against very similar chemicals to demonstrate their selectivity. These alternate chemicals were linked to a protein so that they could be bound to an ELISA microtiter plate. Bar graphs of the results (eight replicates) are presented on pages 13 and 14. Excellent differentiation is shown between the different chlorobenzene isomers. The higher cross-reactivity to 2,4-DNB and 2-AF may have resulted from the carrier proteins (these two chemicals were linked to bovine gamma globulin, which was the immunizing carrier protein for 4-CA). Similar testing will be conducted on the antibodies to 2,4-dichloroaniline and 3,4-dichloroaniline after the supernatants are concentrated.

### **Adaptation of ELISA to Sediments**

The original proposal specified the use of activated carbon as a model sediment, but it was found to be unsuitable. The substituted model sediment was crushed microtiter styrene plates (sieved to obtain a fairly uniform particle size). Crushed microtiter plates were chosen because they are the standard solid phase for ELISAs. The protocol was refined on the styrene chips, and then extended to more realistic particles -- crushed brick and builders sand. Brick was chosen as a model for clay, without the handling difficulties of the fine clay powders. The next steps in the research will be examining smaller particle sizes and mixed particle size distributions.

The sediment ELISA data presented in this report were conducted in 1.5 ml polypropylene microcentrifuge tubes. All liquid additions and withdrawals were made with syringes, therefore eliminating sample loss resulting from opening the tubes. The sample mass can be weighed either before or after immunoassay analysis.

Contaminated chips were made by soaking in a pH 7 buffer solution containing high concentrations of chlorobenzyl compounds (0.05 to 0.5 molar). To increase sorption opportunity, the chips were tumble-mixed in the solutions overnight. The chips continue to soak in the contaminating solutions until needed.

Chips were withdrawn from the soaking solutions and placed in microcentrifuge tubes for each ELISA experiment. Nonspecific binding of the antibodies was prevented by soaking the contaminated (or control) chips in a 30 g/l skim milk/tris solution. Some experiments were conducted to see whether a lower molecular weight blocking agent (L-histidine) than skim milk would improve precision of the procedure. These investigations are continuing.

Immunoassay steps (with multiple wash steps in between) were mouse anti-chloroaniline, goat anti-mouse IgG-biotin or goat anti-mouse IgM-biotin, streptavidin-horseradish peroxidase, chromogen substrate (AZBT<sup>1</sup> + H<sub>2</sub>O<sub>2</sub> in pH 4.1 citrate buffer). The AZBT chromogen was chosen because the end point is a brilliant blue-green: different concentrations were easily distinguished by eye. No stop step was necessary because the chromogen was physically separated from the enzyme by syringe withdrawal. The color was read in a spectrophotometer at 405 nm. All experiments included empty tubes, and clean

---

<sup>1</sup> 2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)



chips as negative controls. The experiments were incubated in a 37°C incubator for temperature control.

The chip ELISA development also included selectivity testing. Both the chlorobenzenes and chloroanilines were used, as well as related chlorobenzyl compounds (i.e. pentachlorophenol). Preliminary testing on the 6H4y clone (anti-3,4-dichloroaniline) of 1,2-dichlorobenzene and pentachlorophenol sorbed on brick chips is shown as an example of testing data on page 15, 16. Recent data using 9H3h antibodies to 4-chloroaniline are shown on page 16. The 9H3h experiment also examined the skim milk blocking concentrations needed for styrene chips. 30 g/l skim milk solution gave improved (lower) background interference. Contaminant orientation is suspected as the cause of nearly zero concentrations for the 4-chloroaniline chips (the 4-chloroaniline chips were noticeably colored from the soaking solution, which allowed visual verification that sorption had occurred). If the antibodies react to a portion of the contaminant molecule that is physically obscured in the sorption process, there will be no antibody attachment. The various monoclonal antibodies will be screened to find the most appropriate antibody to sorbed chemicals.

#### **Soil Samples and Standards**

Two characterized soil samples (approximately 1 kg each) have been received from the Kansas State University Agricultural Experiment Station. The samples will soon be analyzed for grain size distribution. The two soil samples were obtained so that the soil ELISA can progress to analysis of actual contaminated soil. The soil will be contaminated in the laboratory following the procedures listed above for the chips.

One direct measurement of chip contamination was made using  $^{14}\text{C}$ -labelled 3,4-dichloroaniline. Brick chips (#14 sieve size) were soaked overnight in 0.45 molar solution of 3,4-dichloroaniline (1:1000 dilution of  $^{14}\text{C}$ -labelled contaminant). The chips were rinsed repeatedly to simulate the washing of the ELISA process. Scintillation cocktail (Scintiverse I) was hydrated to form a stiff gel. The chips were suspended in the gel for scintillation. The results of the scintillation experiment resulted in a linear relationship between chip weight and total contamination ( $r = 0.95$ ), yielding a slope of 0.515 mg 3,4-DCA/g brick chips. This experiment will be repeated for 4-chloroaniline and 2,4-dichloroaniline.

#### PUBLICATIONS RESULTING FROM THE RESEARCH EFFORT

An invited paper was solicited from Mossman and DiGiacinto by the Computational Hydraulics Committee of the American Society of Civil Engineers for the Specialty Conference on Estuarine and Coastal Modeling which will be held in Chicago in September 1993. The oral presentation is titled "Use of Immunoassay Techniques to Collect Sediment Quality Data for Model Calibration and Verification."

A manuscript, to be submitted to *Analytical Chemistry*, will include results on the selectivity of the antibodies. The authors will be Mossman, Feldbush and Pilgram, with a proposed title of "Specificity of Murine Antibodies to Chlorinated Benzenes using ELISA."

There have been no manuscripts submitted on the soil immunoassay for peer-reviewed publication since the investigators are seeking patent protection.

## PROFESSIONAL PERSONNEL

Deborah J. Mossman, Ph.D., P.E. (principal investigator) is an assistant professor of Civil Engineering at the University of Missouri-Columbia's Truman Campus in Independence, MO. Dr. Mossman teaches undergraduate and graduate classes in environmental engineering.

Thomas L. Feldbush, Ph.D. (co-principal investigator) is the Associate Dean of Medicine for Research at Northwestern University. Dr. Feldbush conducts research and teaches graduate courses in immunology.

Cynthia J. Baker (graduate research assistant) received her M.S. degree in Civil Engineering in May 1993. Her thesis was titled, "Modification of Immunoassay Technique for Determining Soil Contamination."

Susan K. Pilgram (graduate research assistant) is a M.S. candidate in Civil Engineering, expecting to complete her degree in December 1994. Her thesis research is based on the AFOSR project.

Stephen S. DiGiacinto is a Ph.D. candidate in Civil Engineering. He is a part-time student expecting to finish his degree in 1997. His doctoral research will focus on modifying the soil ELISA to accommodate fine-grained suspended sediments.

## INTERACTIONS

### Papers presented

Field Analysis of Contaminated Sediments by Immunoassay. By Deborah J. Mossman, Cynthia J. Baker, Robin D. Rodriguez, and Thomas L. Feldbush. *Proceedings of Water Forum '92* (Environmental Engineering Division), pages 110-115.

Direct Immunoassay of Pollutants on a Solid Sample. By Deborah J. Mossman, Cynthia J. Baker, Thomas L. Feldbush. Presented at Seventh Annual Hazardous Waste Research Conference, 1-2 June 1992, University of Colorado, Boulder, Colorado.

Selectivity of Monoclonal Antibodies for Chlorinated Benzyl Compounds. By D.J. Mossman, S.K. Pilgram, and T.L. Feldbush. Presented at Eighth Annual Hazardous Waste Research Conference, 25-26 May 1993, Kansas State University, Manhattan, Kansas.

## NEW DISCOVERIES

### Patent Applications

The investigators (Mossman, Feldbush) and two student co-inventors (Baker, Rodriguez) are seeking patent protection on the sediment ELISA protocol. The University of Missouri Patents & Licensing office forwarded an invention disclosure to AFOSR on 4/6/92. Sediment analysis without extractions has a number of attractive aspects, including low cost, rapid testing, and safe reagents. The inventors believe that there is a market for such field testing kits. ELISA sediment testing kits would not replace the need for chromatographic testing (GC, HPLC), but would allow the user to screen the contaminated site and choose the samples for laboratory extraction/chromatography analysis. This screening procedure would also protect field personnel by mapping out "hot spots" of contamination which could be avoided or entered with due care.

A second invention disclosure has been filed with the University of Missouri Patents & Licensing office stemming from the AFOSR project. The disclosure, titled "Visualization of Pollutants Sorbed to Soil Using Fluorescent Immunoassay Techniques" was filed with the University's office on 17 March 1993. The inventors are Mossman, Schwab (professor of

agronomy at Kansas State University), and Ousnamer (student). The fluorescent immunoassay technique for visualization is a simple modification of the traditional fluorescent immunoassay. The immunoassay techniques are well established for histological staining of tissue samples. The combination of immobilization and visualization techniques will allow the user to determine the locale of soil sorption by the target chemical. The method could be used for determining the depth of pesticide penetration or whether the analyte binds to roots or soil particles. Given the proper viewing device, this method could be taken to the field and used to monitor pesticide usage at many locations in agricultural use.

#### Additional Research Avenues

The soil immunoassay is being applied to suspended sediments, as a result of research conducted on the AFOSR grant. It is presently difficult to measure the extent of suspended solids contamination in natural waters because of the low sample mass. The ELISA technology's high sensitivity can be exploited to collect these data. This research angle is of particular interest to persons modeling contaminant transport in natural waters, i.e. Great Lakes. No data has yet been collected on the suspended solids ELISA.

The soil immunoassay can also be used to visualize soil contamination. Preliminary experiments used 2,4-dinitrobenzene sulfonate as the model contaminant, and fluorescein-labelled antibodies. The samples were examined under fluorescent microscopy. The uncontaminated brick chips were easily and dramatically distinguished from contaminated ones. These experiments were documented photographically.

## RELATED PROPOSALS

An AASERT proposal was submitted to AFOSR (proposal #93-NL-155) to add another graduate research assistant to the currently funded project. If funded, the AASERT student will develop the fluorescent immunoassay visualization techniques for examining soil contamination.

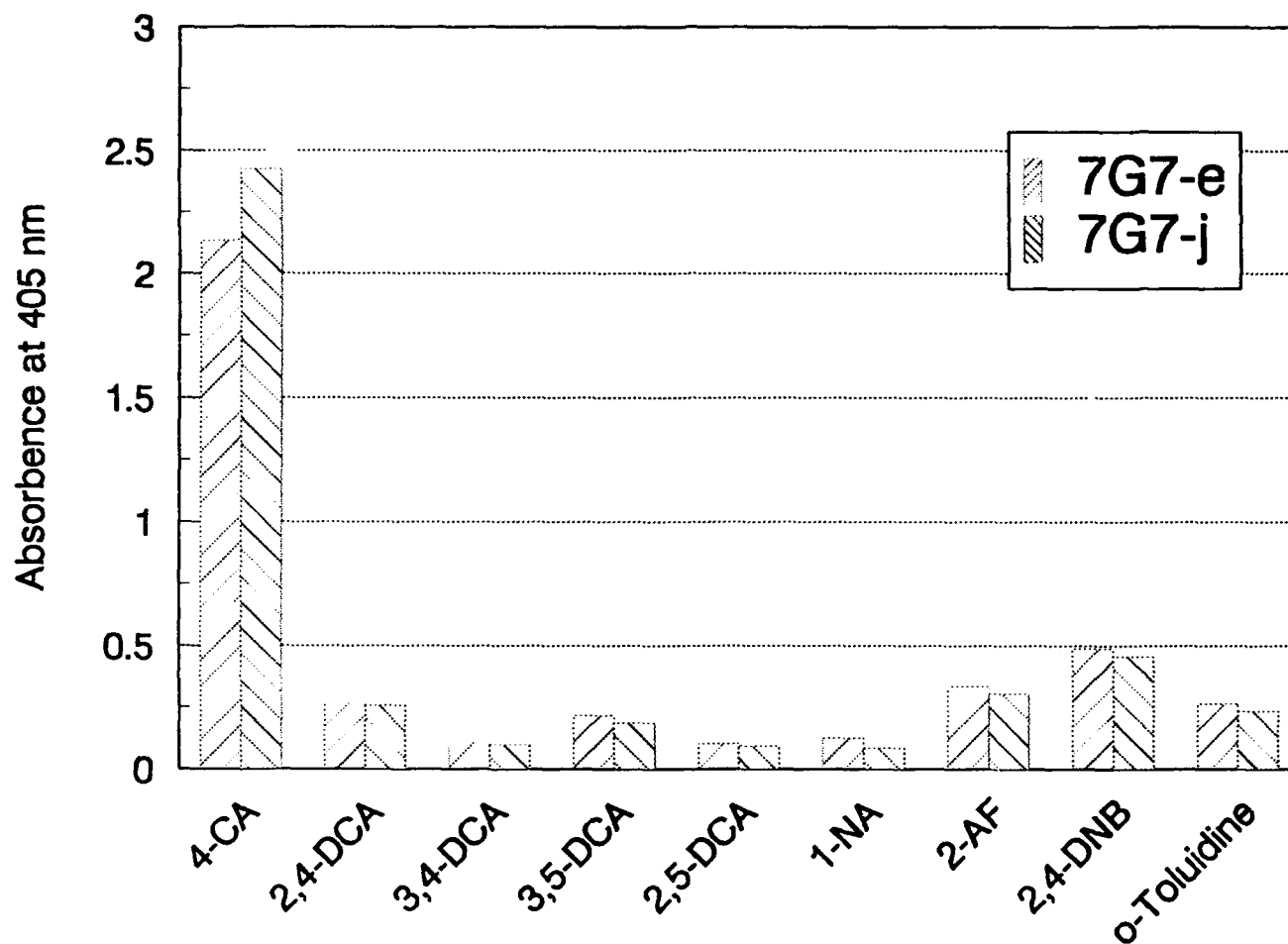
A DEPSCoR proposal was submitted to DoD, titled "Research Equipment Support for Immunoassay Research Applications to Contaminated Sediment Testing," to replace and augment laboratory support equipment related to the soil immunoassay research.

May 27, 1993

# Monoclonal Antibody Detection of Chlorinated Benzenes on Contaminated Sediments

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CIC Core Facility Fusions	.....	.....	.....	.....
Colony Selection	.....	.....	.....	.....
Colony Expansion	.....	.....	.....	.....
Sediment Assay	.....	.....	.....	.....
Create standards	.....	.....	.....	.....
Verify technique	.....	.....	.....	.....
Present results	.....	.....	.....	.....
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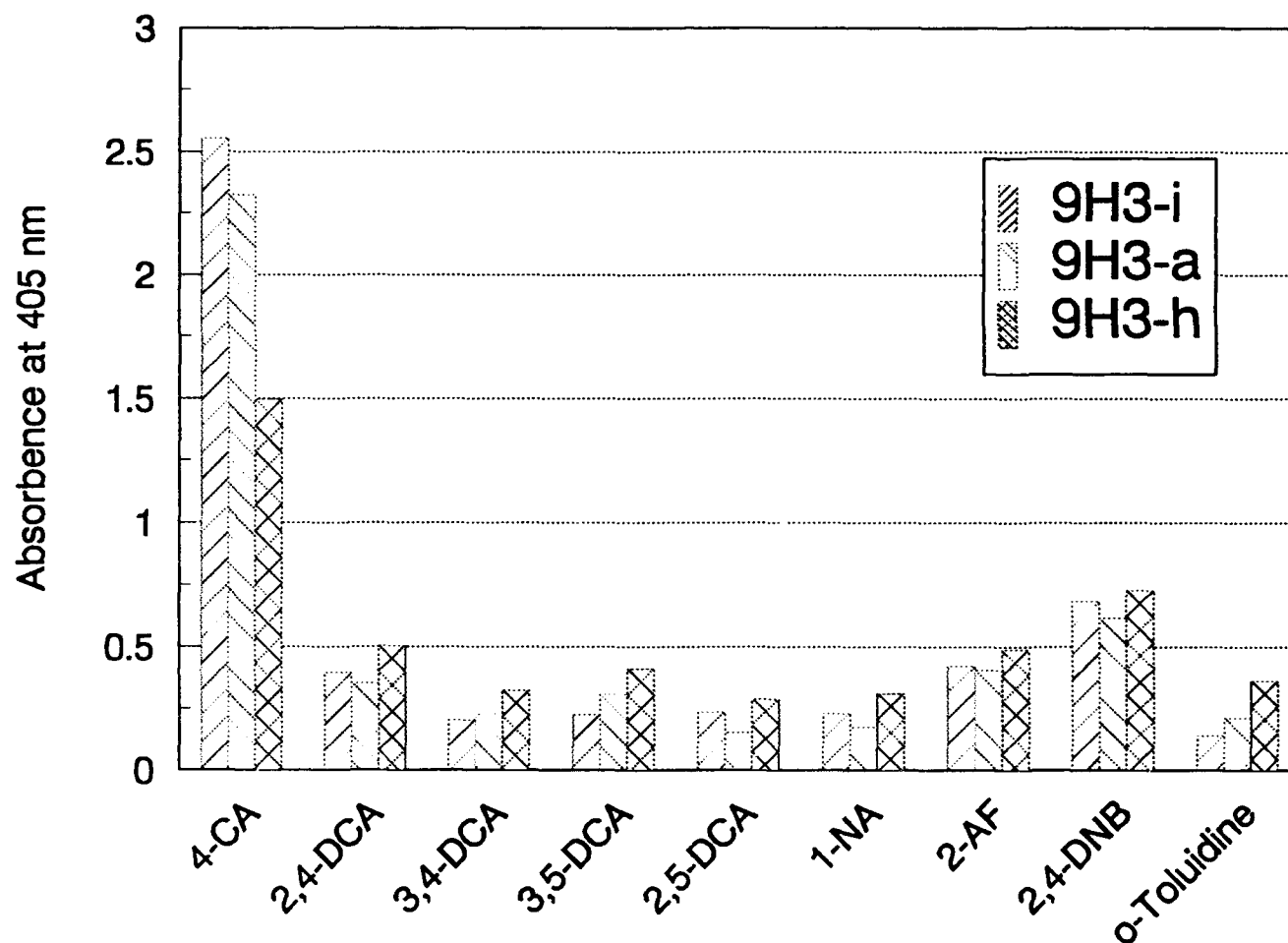
## EXPERIMENTAL RESULTS - 5/20/93



Cross-reactivity study on antibodies to 4-chloroaniline (4-CA). The alternate antigens (linked to proteins) are 2,4-dichloroaniline (2,4-DCA), 3,4-dichloroaniline (3,4-DCA), 3,5-dichloroaniline (3,5-DCA), 2,5-dichloroaniline (2,5-DCA), 1-naphthylamine (1-NA), 2-amino-fluorene (2-AF), 2,4-dinitrobenzene sulfonate (2,4-DNB), and *o*-toluidine.

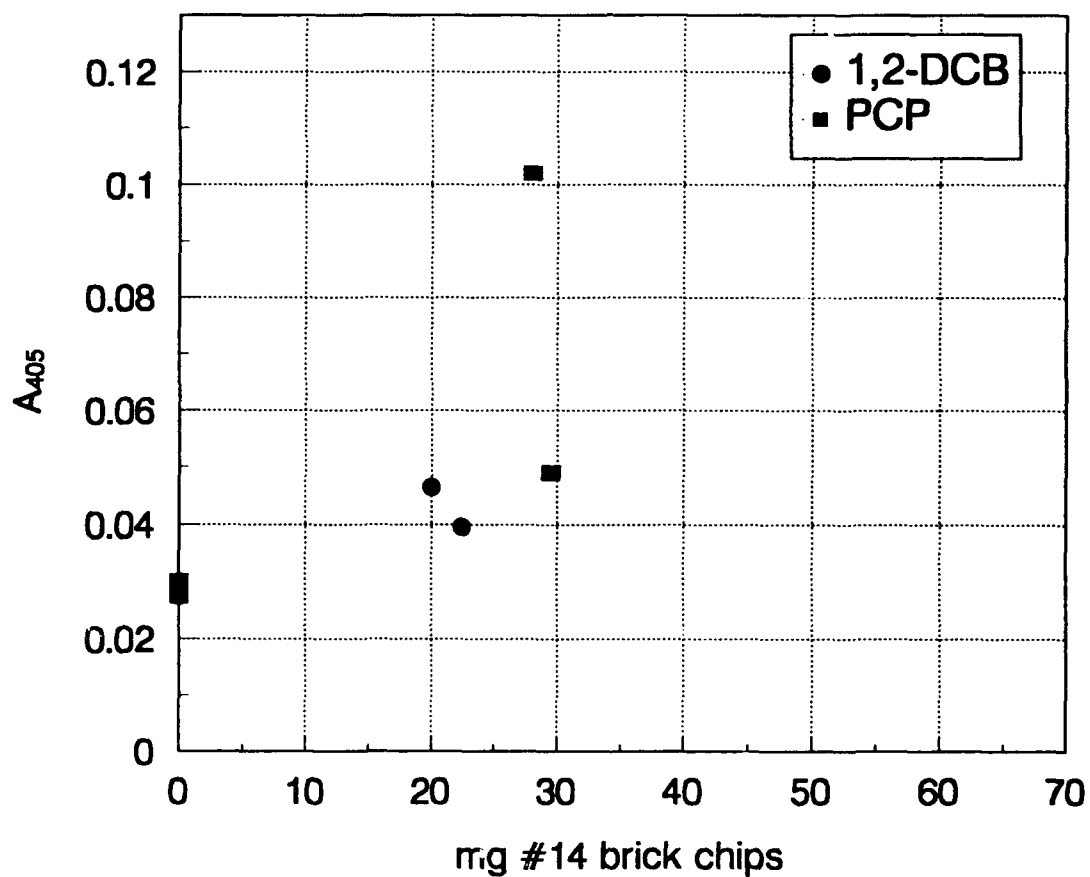


## EXPERIMENTAL RESULTS - 5/20/93



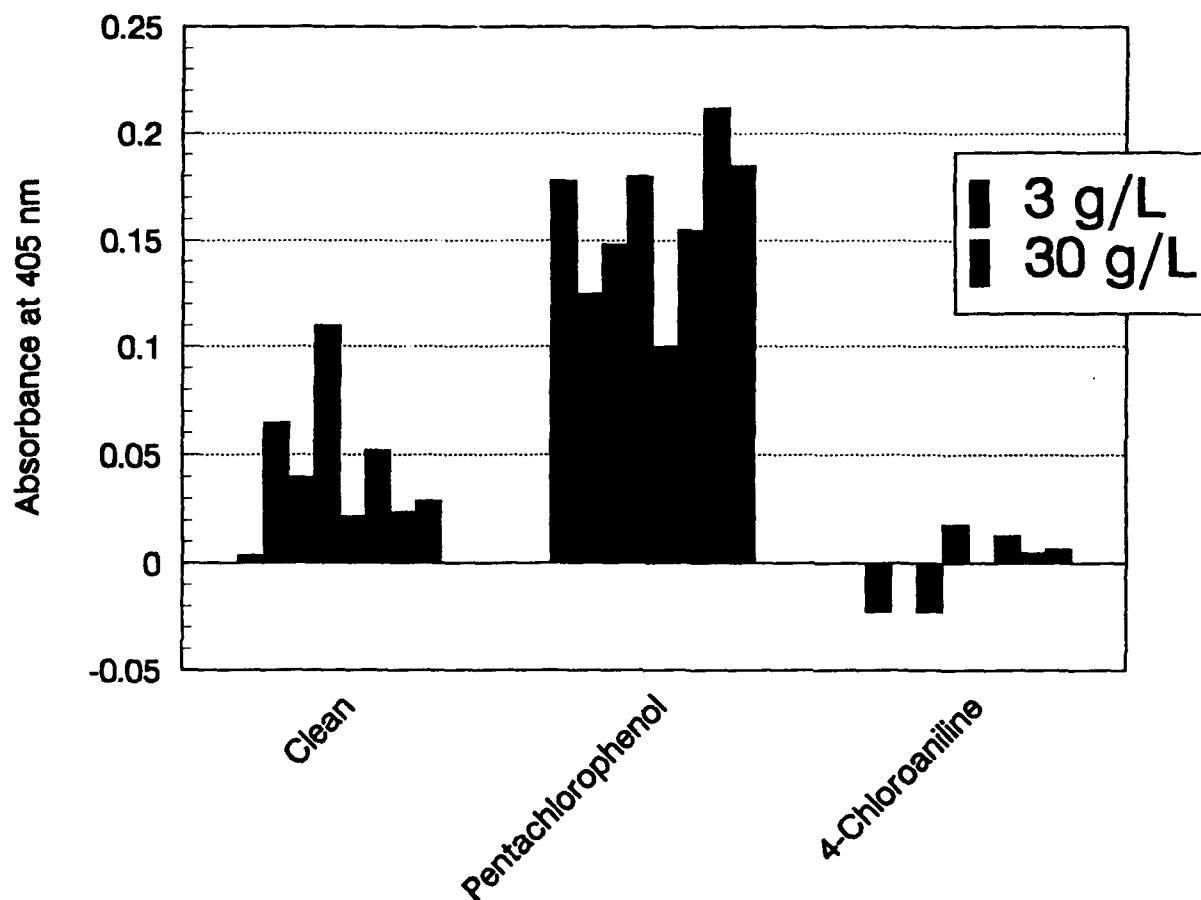
Cross-reactivity study on antibodies to 4-chloroaniline (4-CA). The alternate antigens (linked to proteins) are 2,4-dichloroaniline (2,4-DCA), 3,4-dichloroaniline (3,4-DCA), 3,5-dichloroaniline (3,5-DCA), 2,5-dichloroaniline (2,5-DCA), 1-naphthylamine (1-NA), 2-amino-fluorene (2-AF), 2,4-dinitrobenzene sulfonate (2,4-DNB), and *o*-toluidine.

## EXPERIMENTAL RESULTS - 1/29/93



ELISA using 6H4y monoclonal antibodies to 3,4-dichloroaniline on #14 brick chips contaminated with 1,2-dichlorobenzene and pentachlorophenol.

## EXPERIMENTAL RESULTS - 5/27/93



ELISA using 9H3h monoclonal antibodies to 4-chloroaniline on #14 styrene chips contaminated with 4-chloroaniline and pentachlorophenol. Each bar represents one sample. Two different concentrations of skim milk block were used, and are indicated in the legend.